PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/10

A2

(11) International Publication Number: WO 96/18731

(43) International Publication Date: 20 June 1996 (20.06.96)

(21) International Application Number: PCT/GB95/02893

(22) International Filing Date: 12 December 1995 (12.12.95)

(30) Priority Data:
9425138.6 12 December 1994 (12.12.94) GB

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(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ISOLATION OF NUCLEIC ACID

(57) Abstract

The present invention provides a method of isolating nucleic acid from a sample, said method comprising contacting said sample with a detergent and a solid support, whereby soluble nucleic acid in said sample is bound to the support, and separating said support with bound nucleic acid from the sample. Where the method of the invention is used to isolate DNA, it may conveniently be coupled with a further step to isolate RNA from the same sample.

Isolation of Nucleic Acid

The present invention relates to the isolation of nucleic acid, and especially to the isolation of DNA or RNA from cells.

The isolation of DNA or RNA is an important step in many biochemical and diagnostic procedures. For example, the separation of nucleic acids from the complex mixtures in which they are often found is frequently necessary before other studies and procedures eg. detection, cloning, sequencing, amplification, hybridisation, cDNA synthesis etc. can be undertaken; the presence of large amounts of cellular or other contaminating material eg. proteins or carbohydrates, in such complex mixtures often impedes many of the reactions and techniques used in molecular biology. In addition, DNA may contaminate RNA preparations and vice versa. Thus, methods for the isolation of nucleic acids from complex mixtures such as cells, tissues etc. are demanded, not only from the preparative point of view, but also in the many methods in use today which rely on the identification of DNA or RNA eg. diagnosis of microbial infections, forensic science, tissue and blood typing, detection of genetic variations etc.

In RNA identifications it is important for a conclusive diagnosis to be certain that the detected sequence is derived from an RNA molecule and not from genomic DNA contamination in the sample. For this reason, methods for the separation of RNA from DNA are important. Also, for RNA isolation rapid methods are required since RNA molecules usually are very unstable and rapidly degraded by RNases present in cells and body fluids. The quality of the RNA is probably the most important factor in determining the quality of the final results in protocols utilising mRNA, especially for cDNA synthesis. It is important to avoid DNA contamination

quality mRNA is an important step in the analysis of gene structure and gene regulation.

Most eukaryotic mRNAs have a poly(A)tail, typically about 50 to 300 nucleotides long. Such mRNA is referred to as polyadenylated or poly(A)$^+$ mRNA. In separating this polyadenylated RNA from the non-adenylated RNA which accounts for 95% or more of a cell's total RNA, advantage is taken of this poly(A) tail and some type of affinity separation directed toward the poly(A) tail is performed. The conventional technology has involved purification of total RNA as a first step and selection of poly(A)$^+$ RNA by affinity chromatography using oligo(dT)-cellulose as the second step. This strategy, is rather time-consuming and labour-intensive. An alternative strategy for mRNA purification is to use oligo(dT) linked to solid supports such as microplates, latex, agarose or magnetic beads.

Over the past four years it has become increasingly popular to employ a magnetic bead assisted strategy for poly(A)$^+$ RNA selection since such beads have proven to be favourable in mRNA manipulations. In many approaches, the yield and the quality of the products depends on how rapidly the mRNA can be purified from nucleases and other contaminants. By using the magnetic bead separation technology, pure, intact poly(A)$^+$ RNA can be obtained rapidly either from total RNA preparations or more importantly, directly from crude lysates of solid tissues, cell or body fluids. The entire procedure can be carried out in a microfuge tube without phenol extractions or ethanol precipitations.

One approach common in RNA purification, which may be used in conjunction with the solid phase approach is to carry out the lysis of the biological material and the subsequent hybridisation to oligo dT in LiCl and LiDS/SDS buffers, thereby avoiding extra steps such as phenol extraction or proteinase-K digestion. The whole direct mRNA isolation takes approximately 15 minutes and

which is necessary following alcohol precipitation and the problems with dissolving nucleic acids, are also known to lead to artefacts in enzyme-based procedures such as PCR. Since such procedures are now a mainstay of molecular biology, there is a need for improved methods of nucleic acid isolation, and particularly for methods which are quick and simple to perform and which avoid the use of chaotropic agents or alcohol precipitation. There is also a need for a method which allows for differentiation between RNA and DNA and permits a separate isolation of both types of nucleic acid from the same sample. The present invention seeks to provide such methods.

In particular, it has now been found that nucleic acid may be isolated from a sample in a form suitable for amplification or other downstream processes, by a simple and easy to perform procedure which involves treating the sample with detergent and allowing the nucleic acid to bind to a solid support, whereupon the nucleic acid may be readily separated from the sample, eg. by removal of the support. The binding of the nucleic acid is independent of its sequence.

In one aspect, the present invention thus provides a method of isolating nucleic acid from a sample, said method comprising contacting said sample with a detergent and a solid support, whereby soluble nucleic acid in said sample is bound to the support, and separating said support with bound nucleic acid from the sample.

The nucleic acid may be DNA, RNA or any naturally occurring or synthetic modification thereof, and combinations thereof. Preferably however the nucleic acid will be DNA, which may be genomic, or, cDNA, and single or double stranded or in any other form.

Where the method of the invention is used to isolate DNA, it may conveniently be coupled with a further step to isolate RNA from the same sample. The

heating, for example using a microwave oven (Banerjee, S.K. et al., 1995, Biotechniques 18: 769-773). Also, certain more compact tissues may require enzyme treatment, for example using proteinase K to obtain sufficient release of nucleic acid. The various components are mixed and simply allowed to stand for a suitable interval of time to allow the nucleic acid to bind to the support. Conveniently, if other agents such as enzymes eg. proteinase K are being used, they may be included in with the detergent. The support is then removed from the solution by any convenient means, which will depend of course on the nature of the support, and includes all forms of withdrawing the support away from the sample supernatant, or vice versa, for example centrifugation, decanting, pipetting etc.

The conditions during this process are not critical, and it has been found convenient, for example, simply to mix the sample with the detergent in the presence of a solid phase, and allow it to stand at room temperature, for 5 to 20 minutes, before separating. As mentioned above, the reaction time is not critical and as little as 5 minutes is often enough. However, if convenient, longer periods may be used, eg. 0.5 to 3 hours, or even overnight. Mixing can be done by any convenient means, including for example simple agitation by stirring or vortexing. Also, if desired, higher or lower temperatures may be used, but are not necessary.

The detergent may be any detergent, and a vast range are known and described in the literature. Thus, the detergent may be ionic, including anionic and cationic, non-ionic or zwitterionic. The term "ionic detergent" as used herein includes any detergent which is partly or wholly in ionic form when dissolved in water. Anionic detergents have been shown to work particularly well and are preferred. Suitable anionic detergents include for example sodium dodecyl sulphate (SDS) or other alkali metal alkylsulphate salts or

10mM EDTA
1% LiDS

The detergent functions in the method to lyse the nucleic acid containing material, eg. the cells and nuclei to release the nucleic acid. The detergent is also believed to help to disrupt the binding of proteins, eg. DNA-binding proteins, to the nucleic acid and to reduce the problem of contaminants in the sample sticking to the solid support.

The solid support may be any of the well known supports or matrices which are currently widely used or proposed for immobilisation, separation etc. These may take the form of particles, sheets, gels, filters, membranes, fibres, capillaries, or microtitre strips, tubes, plates or wells etc.

Conveniently the support may be made of glass, silica, latex or a polymeric material. Preferred are materials presenting a high surface area for binding of the nucleic acid. Although not wishing to be bound by theoretical considerations, it is believed that the nucleic acid binding process may be assisted by the nucleic acid "wrapping around" the support. Such supports will generally have an irregular surface and may be for example be porous or particulate eg. particles, fibres, webs, sinters or sieves. Particulate materials eg. beads are generally preferred due to their greater binding capacity, particularly polymeric beads.

Conveniently, a particulate solid support used according to the invention will comprise spherical beads. The size of the beads is not critical, but they may for example be of the order of diameter of at least 1 and preferably at least 2 μm, and have a maximum diameter of preferably not more than 10 and more preferably not more than 6 μm. For example, beads of diameter 2.8 μm and 4.5 μm have been shown to work well.

Monodisperse particles, that is those which are

DYNABEADS, are particularly suited to use in the present invention.

Functionalised coated particles for use in the present invention may be prepared by modification of the beads according to US patents 4,336,173, 4,459,378 and 4,654,267. Thus, beads, or other supports, may be prepared having different types of functionalised surface, for example positively charged or hydrophobic. Weakly and strongly positively charged surfaces, weakly negatively charged neutral surfaces and hydrophobic surfaces eg. polyurethane-coated have been shown to work well.

It is also possible to use solid supports which have been modified to permit the selective capture of desired cells, viruses etc. containing the nucleic acid. Thus for example, supports carrying antibodies, or other binding proteins, specific for a desired cell type may be used. This may introduce a degree of selectivity to the isolation of the nucleic acid, since only nucleic acid from a desired target source within a complex mixture may be separated. Thus for example, such a support may be used to separate and remove the desired cell type etc. from the sample, following which, the detergent is added to achieve lysis, release of the nucleic acid, and binding to the support.

The preparation of such selective cell capture matrices is well known in the art and described in the literature.

Likewise, the support may be provided with binding partners to assist in the selective capture of nucleic acids. For example, complementary DNA or RNA sequences, or DNA binding proteins may be used, or viral proteins binding to viral nucleic acid. The attachment of such proteins to the solid support may be achieved using techniques well known in the art.

Although not necessary, it may be convenient to introduce one or more washing steps to the isolation

separation step, for example by addition of an RNAase or an alkali such as NaOH.

Alternatively, as mentioned above, the method of the invention may be used to separate sequentially DNA and RNA from the sample. It may also be used to remove DNA from a sample in an RNA purification procedure.

Conveniently, the sequential separation may take place using two different solid phases, for example solid supports which can differentiate between DNA and RNA. Thus, such a method may comprise carrying out a first step separation to isolate DNA as described above. A further solid support can then be added to the sample to capture the RNA remaining in the sample, either by using a solid support that can bind the RNA or any remaining nucleic acid, or a solid support that can capture specific RNA molecules (eg. by carrying a complementary nucleic acid probe), or a subset of RNA molecules eg. polyadenylated RNA. In this way it is possible rapidly to isolate and separate DNA and RNA or subsets of both from the same sample. This may be useful, for example by measuring the isolated DNA to estimate the amount of cells used for RNA extraction, which will give a reference between different samples.

However, the DNA isolation procedure of the invention may also readily be combined, as a preliminary step, with other conventional RNA purification procedures, for example DNA isolation with detergent according to invention may be carried out before a selective RNA precipitation step, for example using LiCl or before RNA separation using GTC and sarkosyl.

In a representative procedure, the sample is lysed in the presence of detergent and the DNA is allowed to bind to a solid support, whereupon the DNA may readily be separated from the sample by removal of the support. If desired, the DNA can rapidly and easily be further handled for amplification or other downstream processes. The RNA may then be isolated. This can be by a solid

Maximum absorbance (0.427) at 257.6 nM; minimum absorbance (0.292) at 236.4 nM; at a threshold of 0.100;

Figure 2 shows gel electrophoresis of a sample of DNA isolated as described in Example 1 (lane 1: isolation; lane 2: λ Hind III (molecular weight marker));

Figure 3 shows agarose gel electrophoresis of the PCR product of Example 2 (lane 1: PCR product; lane 2: λ Hind III; lane 3: negative PCR control); and

Figure 4 shows agarose gel electrophoresis of the PCR product of Example 5 (lane 1: λ Hind III; lanes 2 and 3: isolations A and B respectively; lanes 4 and 5: negative control; lane 6: λ Hind III).

Figure 5 show the comparison between traditionally isolated DNA and DNA isolated with Dynabeads DNA DIRECT. Panel I shows the amount of genomic DNA isolated from 10 μl of whole blood with Dynabeads DNA DIRECT including the optional elution step (lanes 1 and 2), with Dynabeads DNA DIRECT with the elution step omitted (lanes 3 and 4), and with traditional DNA isolation (lanes 5 and 6). The molecular weight marker in lane 7 is λ HindIII. Panel II shows the integrity of DNA isolated by Dynabeads DNA DIRECT. Lanes 1 and 2 show AMXY PCR from 20 ng of DNA isolated with Dynabeads DNA DIRECT from a male and female donor respectively. Lanes 4 and 5 show AMXY PCR from 200 ng DNA isolated by traditional methods from a female and a male donor respectively. Lane 3 is the negative control.

Figure 6 shows the reproducibility of Dynabeads DNA DIRECT. The figure shows five independent Dynabeads DNA DIRECT isolations from each of two donors. Half of the DNA obtained from 10 μl of blood is shown in the upper part of the figure, 20% of the product from PCR reactions started with 10% of the isolated DNA is shown in the lower part of the figure. Molecular weight markers are λ HindIII (lanes marked M) or 100 bp ladder (lane marked L).

product from PCR reactions started with 10% of the isolated DNA is shown in the lower part of the figure. Panel II shows two Dynabeads DNA DIRECT isolations from 4 x 10^5 Daudi cells. One tenth of the DNA obtained is shown in the upper part of the figure, 20% of the product from PCR reactions started with 1 µl of a total of 120 µl isolated DNA is shown in the lower part of the figure. The molecular weight marker is λ HindIII for the genomic DNA and 100 bp ladder for the PCR products.

Figure 10 shows Dynabeads DNA DIRECT from formalin fixed, paraffin embedded material. Lane A is 20% of the PCR product from a reaction started with DNA isolated by DNA DIRECT from a formalin fixed, paraffin embedded section of liver. Lane M is molecular weight marker (100 bp ladder), lane B is positive control (PCR from 20 ng human DNA), and lane C is negative control (PCR from water).

Figure 11 shows Dynabeads DNA DIRECT for mRNA purification. mRNA was isolated from 1 million Daudi cells per sample with Dynabeads Oligo$(dT)_{25}$ after removal of DNA with Dynabeads DNA DIRECT. Increasing amounts of DNA DIRECT Dynabeads were used to remove genomic DNA; 1 mg in lane 1 and 2; 2 mg in lane 3 and 4; 5 mg in lane 5 and 6 and 10 mg in lane 7 and 8. Lane 9 and 10 are controls where no DNA was removed before direct mRNA purification. The extra bands on the top of the picture show contaminating genomic DNA in lane 9 and 10. The two strong bands in all lanes represent ribsomal RNA.

Figure 12 shows the results of DNA isolation and PCR amplification from (A) bacteria, (B) fungi, (C) algae and (D) plants. For all samples, DNA was isolated with 200 µl DNA DIRECT (one sample test) and 20% of the isolated DNA and 10% of the PCR products were analysed by agarose electrophoresis. For bacteria, 2.5% of the isolated DNA was used per PCR reaction, for the other samples 5% was used. 16S rRNA regions were amplified from bacterial genomic DNA and from algae chloroplast

Example 1

DNA isolation from cell culture

$4x10^6$ HL60 cells were washed twice in PBS and pelleted. The pellet was dissolved in 10 µl PBS, and 1 mg of Dynabeads® M-280* obtainable by autoclaving a suspension of Dynabeads® M-280 tosylactivated (available from DYNAL A/S, Oslo, Norway) in water) resuspended in 0.1 ml lysis buffer [1% SDS/10 mM TrisCl pH 8.0/1 mM EDTA] was added. This was followed immediately by the addition of 1 ml lysisbuffer, and the suspension was incubated for 5 minutes at room temperature, after which the Dynabeads®, with bound DNA was attracted to a magnet and the liquid phase removed. The solid phase was then washed twice with 1 ml washing buffer [50 mM NaCl/10 mM TrisCl pH 8.0/1 mM EDTA]. Finally, the beads, with bound DNA, were resuspended in 0.1 ml water, and incubated for 5 minutes at 65°C. The beads were attracted to a magnet, and the liquid phase withdrawn. The liquid phase was then analyzed for its DNA content. Results from an optical density scan (Fig. 1) are in accordance with pure DNA. The OD_{260}/OD_{280} ratio is 1.72; pure DNA in water or TE has a ratio of 1.7 - 1.9. With pure DNA, the concentration can be determined from the OD_{260} of the solution. A 50 µg/ml solution has OD_{260} = 1.0. From the OD_{260} measurement (Table 1) of 0.436 (0.1 ml total volume, 10 mm lightpath), the yield can be calculated to 2.18 µg DNA, 82% of the 2.67 µg that was the estimated DNA content of the starting material. Gel electrophoresis of a sample of the isolated DNA (Fig. 2) shows that most of it is in a high molecular weight form (>20 kb).

Example 3

Example 1 was repeated using the following combination of lysisbuffers and washing buffers, and the following results were obtained:

(where +++ indicates very good DNA isolation)

Lysis buffer	Washing buffer	Result
2% SDS	50 mM NaCl/1 x TE	+++
2% SDS/1 x TE	50 mM NaCl/1 x TE	+++
2% SDS/1 x TE/10 mM NaCl	50 mM NaCl/1 x TE	+++
5% SDS	50 mM NaCl/1 x TE	+++
5% SDS/1 x TE	50 mM NaCl/1 x TE	+++
5% SDS/1 x TE/10 mM NaCl	50 mM NaCl/1 x TE	+++
1% LiDS/10 x TE/0.5 M LiCl	50 mM NaCl/1 x TE	+++
1% LiDS/10 x TE/0.5 M LiCl	150 mM LiCl/1 x TE	+++
5% LiDS	150 mM LiCl/1 x TE	+++
5% SDS	150 mM LiCl/1 x TE	+++
1% Sarcosyl	150 mM LiCl/1 x TE	+++

1 x TE is 10 mM TrisCl pH 8.0/1 mM EDTA, 10 x TE is 100 mM TrisCl pH 8.0/10 mM EDTA

Example 4

Following the procedure of Example 1, similar results may be achieved using Dynabeads® M-450 uncoated (Dynal A/S, Oslo, Norway)

10 µl of the same blood sample were performed using Dynabeads DNA DIRECT (kit, commercially available from Dynal AS, Oslo, Norway, containing beads equivalent to Dynabeads® M-280* as described in Example 1). The DNA from two of the isolations was eluted for 5 minutes at 65°C, while the DNA from the other two isolations was left in the presence of the Dynabeads. All the DNA from the four Dynabeads DNA DIRECT isolations was loaded onto an agarose gel, as was 0.2% of traditionally isolated DNA. The fraction of the traditionally isolated DNA loaded corresponds to the yield from 10 µl of blood (0.2% of 5 ml).

Traditional DNA isolation was performed according to the method of John and coworkers (John, S.W.M., G. Weitzner, R. Rosen and C.R. Scriver. 1991. A Rapid Procedure for Extracting Genomic DNA from Leukocytes. Nucl. Acid. Res. 19(2):408).

With Dynabeads DNA DIRECT, lysis of the blood was obtained by mixing 200 µl (one sample test) of Dynabeads DNA DIRECT with 10 µl of blood in a 1.5 ml microcentrifuge tube (200 µg uncoated Dynabeads in Lysis/binding buffer). Lysates were then left on the bench at room temperature for 5 minutes to allow adsorption of genomic DNA to the Dynabeads.

The DNA/Dynabeads complex was attracted to a magnet (Dynal's Magnetic Particle Collector E (MPC-E)), and the lysate was aspirated and discarded.

The complex was then washed twice in washing buffer (one of the kit components) by attracting it to a Dynal MPC and discarding the supernatant. Finally, the complex was resuspended in 10 µl of TE pH 8.0 (provided in the kit).

3') and AMXY-4R (5'-TTCATTGTAAGAGCAAAGCAAACA-3') were added per reaction. PCR was performed on a Perkin Elmer GeneAmp PCR System 9600. PCR conditions for the AMXY amplicon were 4 min at 94°C, 38 X [30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C], 10 min at 72°C.

10 µl of the 50 µl PCR reactions were visualised on ethidium bromide stained 1.5% agarose gels. Electrophoresis was performed in 1 x TAE buffer, and the results were documented with a DS34 Polaroid camera and Polaroid 667 film.

The results of this experiment are shown in panel II of figure 5. The X-Y homologous amelogenin gene is known to be sensitive to DNA degradation (Akane et al 1994, supra). With increasing degradation, the 908 bp long X band gets progressively weaker as compared to the 719 bp long Y band. From panel II of figure 5 it is apparent that the relative strength of the X and Y bands is comparable for DNA isolated with Dynabeads DNA DIRECT and the traditional method, indicating that the degree of degradation is the same with the two methods. The PCR reactions from traditionally isolated DNA gives somewhat more product than does the reactions from DNA DIRECT isolated DNA. The reason for this is that ten times more template is used in the PCR reactions from traditionally isolated DNA than in the PCR reactions from DNA DIRECT isolated DNA.

Lysis/binding buffer:	0.5 M LiCl 1 % LiDS 0.1 M TrisCl pH 7.5 10 mM EDTA 5 mM dithiothreitol (DTT)
Washing buffer:	0.15 M LiCl 10 mM Tris-HCl pH 8.0 1 mM EDTA

concentration of 1 x, dNTPs (Pharmacia) were added to a final concentration of 0.2 mM, and 1 unit of amplitaq (Perkin Elmer) was used per reaction. 5 pmol each of primers GAPDH-Forward (5'-ACAGTCCATGCCATCACTGCC-3') and GAPDH-Reverse (5'-GCCTGCTTCACCACCTTCTTG-3') were added per reaction. PCR was performed on a Perkin Elmer GeneAmp PCR System 9600. PCR conditions for the GAPDH amplicon were 4 min at 94°C, 34 X [30 sec at 94°C, 30 sec at 61°C, 1 min at 72°C], 10 min at 72°C.

Both genomic DNA and PCR products were visualised on ethidium bromide stained 1.5% agarose gels. 10 µl of the 50 µl reaction was loaded on to an agarose gel, as was 50 % of the isolated genomic DNA. Electrophoresis was performed in 1 x TAE buffer, and the results were documented with a DS34 Polaroid camera and Polaroid 667 film.

The results of this experiment are shown in figure 6. No significant variation between the different isolations can be observed. Similar results were obtained with other coagulants as well as from donors with higher white blood cell counts.

EXAMPLE 8

DNA isolation from blood with different anticoagulants

Dynabeads DNA DIRECT (kit, commercially available from Dynal AS, Oslo, Norway) was used to isolate DNA from untreated whole blood as well as blood anticoagulated with EDTA, Citrate or Heparin. From each type of starting material, two separate isolations were performed, with blood from different donors. The buffer components in the kit are as described in example 6.

Lysis of the DNA containing cells from blood was

gel, as was 25 % (50 % from the Heparin samples) of the isolated genomic DNA. Both genomic DNA and PCR products were visualised on ethidium bromide stained 1.5% agarose gels. Electrophoresis was performed in 1 x TAE buffer, and the results were documented with a DS34 Polaroid camera and Polaroid 667 film.

The results of this experiment are shown in figure 7. As the isolations from Heparinized samples were from only 5 µl of blood, using 20 % of the DNA from these isolations as starting material for PCR is comparable to using 10 % from the other isolations, that are all from 10 µl blood. When this is taken into consideration, it is apparent that the type of anticoagulant used does not significantly affect the result.

In the experiment just described, Lithium Heparin was used. In this system, similar results are obtained with Lithium and Sodium Heparin, even though Lithium Heparin has been shown to have inhibitory effects in other systems (Panaccio, M., M. Georgesz and A.M. Lew. 1993. FoLT PCR: A Simple PCR Protocol for Amplifying DNA Directly from Whole Blood. BioTechniques 14(3): 238-243). DNA DIRECT also performs well on blood anticoagulated with ACD (panel II of figure 5) or CPD (data not shown).

Example 9

Isolation of DNA from blood samples stored under different conditions

Dynabeads DNA DIRECT (kit, commercially available from Dynal AS, Oslo, Norway) was used to isolate DNA from EDTA blood from two different donors. What was remaining of the blood samples were then divided into two, one part that was stored at +4°C and one that was

Using Dynabeads DNA DIRECT as described earlier in this example, DNA was isolated from two citrate treated blood samples, and from the same two samples 10 µl was spotted on a plastic surface and allowed to air dry at room temperature. The dried blood spots were transferred to 1.5 ml tubes, 40 µl PBS was added, and the tubes were left at room temperature with gentle agitation for 90 min, before DNA was isolated with Dynabeads DNA DIRECT. From each of the 4 isolations (fresh and dried), 10 % was used as starting material for PCR amplification of the GAPDH amplicon as described in example 8. Both genomic DNA and PCR products were visualised on ethidium bromide stained 1.5% agarose gels. 10 µl of the 50 µl reaction was loaded on the gel, as was 50 % of the isolated genomic DNA. Electrophoresis was performed in 1 x TAE buffer, and the results were documented with a DS34 Polaroid camera and Polaroid 667 film. The results of this experiment are shown in panel II of figure 8. The yield from dried blood is good and the isolated DNA is suitable for PCR.

Example 10

DNA isolation from Bone marrow and Culture cells

DNA ISOLATIONS FROM BONE MARROW

1, 2, and 5 µl of heparinized bone marrow from each of two healthy donors were used as starting material for DNA isolation with DNA DIRECT. The buffer components are as described in example 6. Lysis of the bone marrow was obtained by mixing 200 µl (one sample test) of Dynabeads DNA DIRECT with 1-5 µl of heparinized bone marrow in a 1.5 ml microcentrifuge tube. Lysates were then left on the bench at room temperature for 5 minutes to allow adsorption of genomic DNA to the Dynabeads.

DNA ISOLATION FROM CULTURED CELLS

Two samples of 4 x 10^5 Daudi cells were used as starting material for DNA isolation with DNA DIRECT. DNA isolation from 4 x 10^5 cultured cells (cell line Daudi) was performed as described above, except that 1 ml (five sample tests) of Dynabeads DNA DIRECT was used. Accordingly, the washing steps were performed in 1 ml washing buffer. The DNA/Dynabeads complex was resuspended in 120 µl TE, and as for bone marrow, no elution step was performed after the resuspension.

From each of the isolations, 1 µl of a total of 120 µl was used as starting material for PCR amplification of the GAPDH amplicon, as described in Example 8.

Both genomic DNA and PCR products were visualised on ethidium bromide stained 1.5% agarose gels. 10 µl of the 50 µl reaction was loaded on to an agarose gel, as was 10 % of the isolated genomic DNA. Electrophoresis was performed in 1 x TAE buffer, and the results were documented with a DS34 Polaroid camera and Polaroid 667 film.

The results of this experiment are shown in panel II of figure 9, demonstrating that at least 120 PCR reactions may be run from an isolation of this scale.

Example 11

Isolation of DNA from a formalin fixed, paraffin embedded section of liver

Dynabeads DNA DIRECT (kit, commercially available from Dynal AS, Oslo, Norway) was used to isolate DNA from a formalin fixed, paraffin embedded section of liver. The buffer components of the kit and the bead concentration

Example 12:

Removal of genomic DNA with DNA DIRECT prior to mRNA isolation.

mRNA was isolated from 1 million Daudi cells per sample. The cells were lysed in 0.75 ml Lysis/binding buffer with DNA DIRECT Dynabeads present in the buffer. The samples were incubated for 5 minutes and the DNA-Dynabead complexes were collected by applying a Dynal MPC-E magnet for 2 minutes. Different amounts of DNA DIRECT beads were used to remove genomic DNA; 1, 2, 5 and 10 mg per sample (Figure 11).

The lysate from each sample was transferred to new tubes with 1 mg Dynabeads Oligo$(dT)_{25}$ according to standard procedure (Dynals mRNA DIRECT kit protocol). The Dynabeads were mixed with the lysate to capture the polyadenylated mRNA by hybridisation for 5 minutes at room temperature. The mRNA-Dynabead complexes were collected with the MPC-E magnet by placing the tubes in the magnetic stand for 2 minutes. The solution was removed and discarded. Washing solution with LiDS (0.75 ml) was added and the beads were washed thoroughly by pipetting up and down. The mRNA-Dynabead complexes were collected with the magnet, and the washing procedure was repeated once with washing buffer with LiDS and twice with washing buffer without detergent. Finally, the purified mRNA was eluted from the Dynabeads in 20 µl 5 mM Tris-HCl pH 7.5 buffer, by incubation at 65° C for 2 minutes. The eluates were analysed by non-denaturing gel electrophoresis in a 1.0 % agarose gel with ethidium bromide. Figure 11 shows the results from this experiment.

The EtBr-staining reveals both double-stranded DNA and rRNA due to secondary structure. The two ribosomal RNA

of Water Research, 1991, Culture collection of algae). 20-200 million bacteria or 450,000 cyanobacteria were used per DNA isolation.

Agar plates containing 2% malt extract was used for mycelia growth and incubated for 14 days at room temperature. Mycelia was isolated by scraping the surface of the agar plates with a spatula. Fungi fruitbodies were obtained from natural populations. In the range of 1-3 mg air dried and 3-20 mg fresh fungi fruitbodies were used per DNA isolation.

Bakers yeast Saccharomyces cerevisiae was obtained from a commercial supplier. Algae were cultured under illumination in IMR-medium for 7 days (Eppley, R et al., 1967, Exp. Mar. Biol. Ecol. 1, 191-208). Fresh leaves from Arabidopsis thaliana and barley (Hordeum vulgare) were picked from young plants (3 weeks old). Epithelia were obtained from perch (Perca fluvatilis) fins. About 1 mg wet weight yeast, 30-100 mg young plant leaves and 100-400 mg perch were used per DNA isolation.

Multicellular tissues with rigid cell walls were mechanically broken to increase DNA yield. Fungi fruitbodies were ground with foreceps for about 2 minutes. Plant leaves were homogenised for 2 minutes in liquid nitrogen with a pestle (Kontes Scientific Instruments, Vineland, New Jersey, USA). For all other samples no mechanical work was required for cell breakage.

DNA isolation

DNA isolations were performed using Dynabeads DNA DIRECT (kit, commercially available from Dynal AS, Oslo, Norway). Lysis of the cells and organisms were obtained by mixing 200 µl of Dynabeads DNA DIRECT (200 µg uncoated Dynabeads in Lysis/binding buffer) with the

methods were used. Algae, vertebrate and bacterial DNA were isolated with the protocol described by Sambrook, J. et al., 1989, supra. Cyanobacteria were homogenized with alumina type A-5 (Sigma Chemicals Co., St. Louis, USA) before isolation to ensure complete lysis. Plant and fungi DNA were isolated with the protocol described by Scot, O.R. and Bendich, A.J., 1994, in "Plant Molecular Biology Manual", page D1: 1-8, Kluwer Academic Publisher, Belgium.

PCR amplifications

For each sample type the reproducibility was tested by using separate DNA isolations, serial DNA dilutions and multiple PCR assays. DNA isolation reagents and PCR reagents were controlled for absence of contamination in each separate experiment. All PCR reactions were performed in a 50 µl reaction volume containing; 15 pmoles primers, 200 µM dNTP, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 1 Unit DynaZyme thermostable polymerase (Finnzymes Oy, Finland) and 0.1-5 µl of isolated DNA. PCR was performed on a Perkin Elmer GeneAmp PCR System 9600.

Amplicons and oligonucleotide primers

All PCR reactions were started with a DNA denaturation step at 94-97°C for 3 to 5 minutes and ended with an extension step at 72°C for 5 minutes.

Bacteria and algae:

The amplicon was a 16S rRNA region corresponding to E. coli base 334 to 939 according to IUD numbering from bacteria and algae chloroplasts (Brosius, J., et al., 1978, Proc. Natl. Acad. Sci., USA, 57, 4801-4805).
Primers: CC 5'-TGTAAAACGACGGCCAGTCCAGACTCCTACGGGAGGCAGC-3'
CD 5'-CTTGTGCGGGCCCCCGTCAATTC-3'

A part of the Barley gene B15C (800 bp) was amplified with the primers 5'-CGGATCCCGTCATCCTCTTCTCGCACCCC-3' and 5'-GGAATTCCCTTCTTGGAGGGCAGGTCGGCG-3'.

Amplification: 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute.

Perch: Mitochondrial D-loop fragment (800-900 bp) was amplified with the primers HV2 described by Hoelzel et al., 1991, Mol. Biol. Evol., 8, 475-493, and the primer 5'-GGTGACTTGCATGTGTAAGTTCA-3'.

Amplification: 30 cycles of 96°C for 1 minute, 52°C for 2 minutes and 72°C for 2 minutes.

The amplified fragments were visualised on ethidium bromide stained 1.5% agarose gels. Electrophoresis was performed in 1 x TAE buffer, and the results were documented with a DS34 Polaroid camera and Polaroid 667 film.

The results of the experiments are shown in Figure 12 and Table 2.

Bacteria: The standard protocol gave DNA yields in the range of 100-1000 ng for the bacteria tested (Fig. 12A). For some Cyanobacteria there was a substantial increase in DNA yield (from 500 ng to more than 1 mg) by improving the lysis with an extra initial incubation step at 65°C for 15 minutes. In all cases, good amplifications were obtained by using 0.25% of the isolated DNA.

Fungi: The highest DNA yield was obtained from dried fruit-bodies (300-500 ng) compared with fresh fruit bodies (100-200 ng) (Fig. 12B). Mycelia gave low DNA recovery probably due to low number of cells per sample. However, in most cases 5% of the isolated DNA was enough

Table 2: DNA isolation and PCR amplification from different organisms

Species[a]			Sample[b]	DNA yield[c]	Amplicon[d] Gen.	Amplicon[d] Org.	PCR product[e]
Bacteria							
Bacillus sereus AH 75[a]	gram pos.		fresh	+++	16S		+++
E. coli NovaBlue[f]			fresh	+++	16S		+++
A. tumefaciens GV 310[g]			fresh	+++	16S		+++
Plankothrix agardhii N-C 29			fresh	+++	16S		+++
P. prolifica N- C320	gram neg.		fresh	+++	16S		+++
Microsystis aruginosa N-C 43			fresh	+++	16S		+++
M. aruginosa N-C 228/1		Cyanobacteria	fresh	+++	16S		+++
Anabaena bory N-C 246			frozen	+++	16S		++
Phormidium sp N-C 177			frozen	+++	16S		++
Aphanizomenon sp N-C 103			frozen	+++	16S		+++
P. hollandica N 5/89		prochlorothrix	frozen	++	16S		+++
Fungi							
Cortinarius sanguineus			d.fruitb.	+++	18S		+++
Cortinarius gentilis			d.fruitb.	nt	18S		+++
Russula insegra		basidiomycetes	f.fruitb.	++	18S		+++
Laccaria bicolor			f.mycel	+	18S		+++
Triharia ochroleuca			f.mycel	nt	18S		+++
Verjahinia calthae		ascomycetes	f.mycel	nt	18S		+++
Peziza vesiculosa			f.mycel	nt	18S		+++
Saccharomyces cevisiae		yeast	fresh	+	18S		+++
Algae							
Gyrodinum aureolum			fresh	+++	18S/16S		+++/+++
Hetrocapsa triquetra			fresh	+++	18S/16S		+++/+++
Scripsiella trochidea		dinoflagellates	fresh	+++	18S/16S		+++/+++
Ceranium strictum			fresh	+++	18S		+++
Chlorella vulgaris			fresh	+++	18S		+++
Clamydomonas reinardii		chlorophyta	fresh	+++	18S		+++
Caülacantus ustulata		phacophyceae	fresh	+++	18S		+++
Chrysochromulina polylepis		crysophyceae	fresh	+++	18S		+++
Plants							
Hordum vulgare (barly)		monocot	leaf	+++	B15C/*trnL*		+++/+++
Arabidopsis thaliana		dicot	leaf	+++	B15C/*trnL*		+++/+++
Vertebrates							
Perca fluvatilis (perch)	fish		ep.	+++	D-loop		+++

[a] A. tumerfaciens = Aghrobacterium tumefaciens,

P. hollandica = Prochlorothrix hollandica.

[b] d.fruitb = dried fruitbodies, f.mycelia = fresh mycelia, ep. = epithelium.

[c] Approximate DNA yields relative to standard phenol/chloroform isolations;

+++: > 80 %, ++: > 10 %, +: > 1 %, nt. = not tested.

[d] Gen. = genomic DNA, Org. = organelle DNA from chloroplasts (algae and plants) and mitochondria (fish).

[e] Amplicons as described in example 13.

10. A method as claimed in any one of claims 1 to 9, wherein the detergent is used in alkaline solution.

11. A method as claimed in any one of claims 1 to 10, wherein the solid support is particulate.

12. A method as claimed in claim 11, wherein the solid support comprises magnetic beads.

13. A method as claimed in any one of claims 1 to 12, wherein the solid support has a hydrophobic surface.

14. A method as claimed in any one of claims 1 to 13, wherein the nucleic acid is eluted from the support, following separation from the sample.

15. A method as claimed in claim 14, wherein the nucleic acid is eluted by heating.

16. A kit for isolating nucleic acid from a sample, comprising a solid support and one or more detergents as defined in any one of claims 1 to 13.

17. A kit as claimed in claim 16, further comprising one or more buffers, salts, lysis agents, chelating agents and/or reducing agents.

18. A kit as claimed in claim 16 or claim 17, further comprising means for isolating RNA.

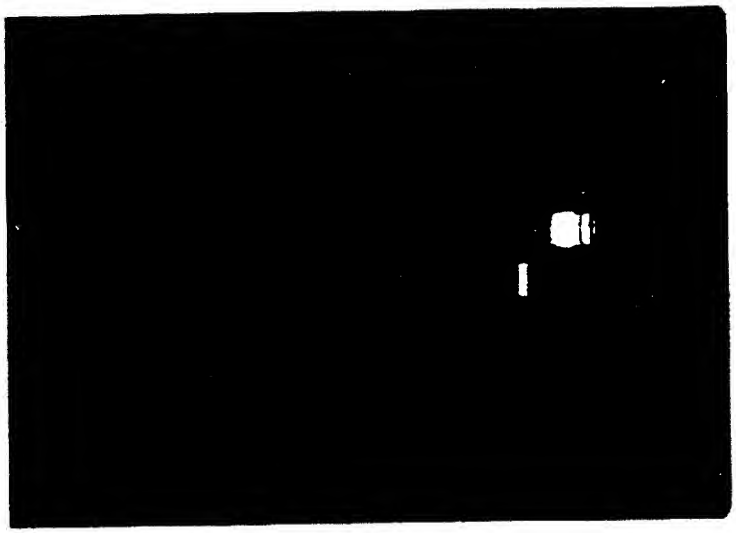

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FIG. 3

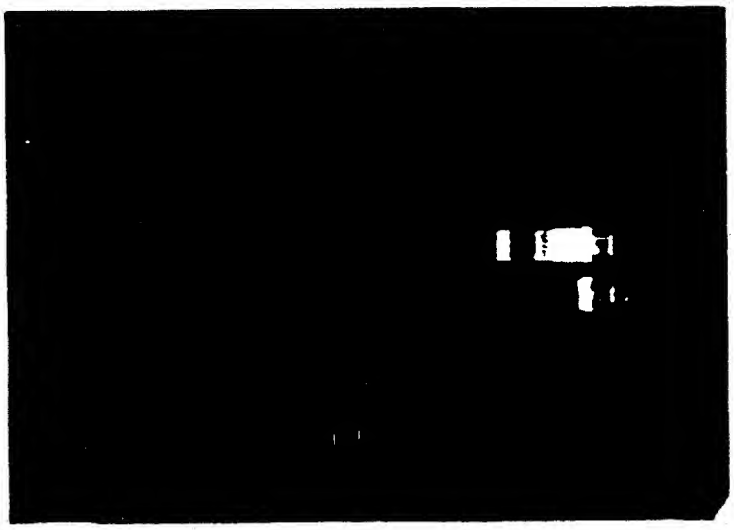

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FIG. 2

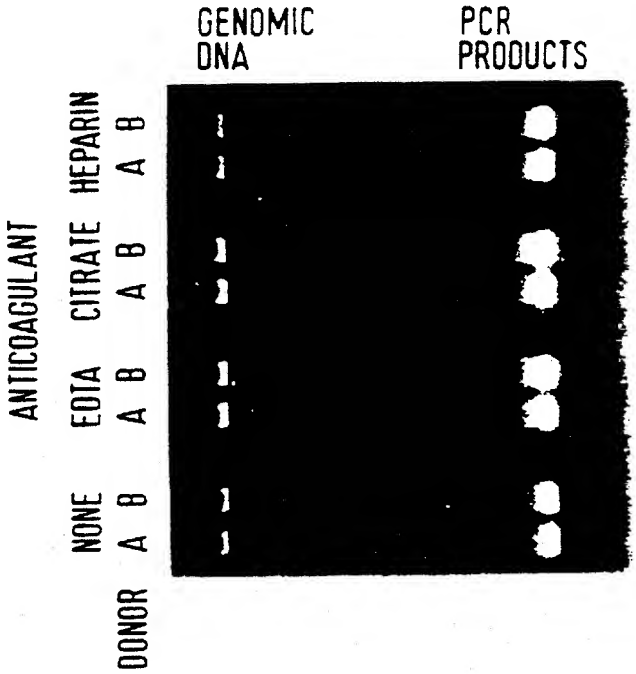
GENOMIC DNA
PCR PRODUCTS
ANTICOAGULANT
NONE EDTA CITRATE HEPARIN
DONOR A B A B A B A B

FIG. 7

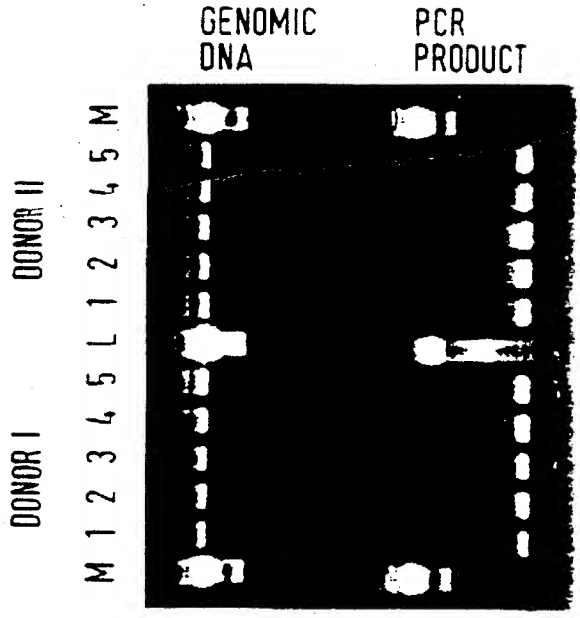
GENOMIC DNA
PCR PRODUCT
DONOR I
DONOR II
M 1 2 3 4 5 L 1 2 3 4 5 M

FIG. 6

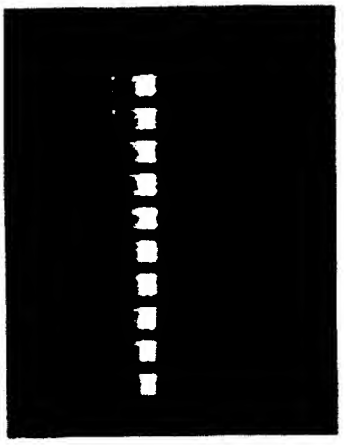

FIG. 11

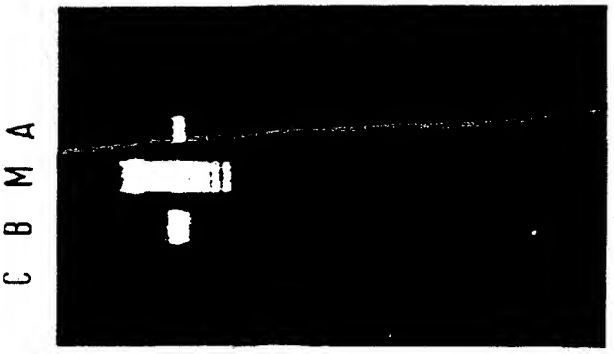

FIG. 10

PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/10 | A3

(11) International Publication Number: WO 96/18731

(43) International Publication Date: 20 June 1996 (20.06.96)

(21) International Application Number: PCT/GB95/02893

(22) International Filing Date: 12 December 1995 (12.12.95)

(30) Priority Data:
9425138.6 12 December 1994 (12.12.94) GB

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(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
12 September 1996 (12.09.96)

(54) Title: ISOLATION OF NUCLEIC ACID

(57) Abstract

The present invention provides a method of isolating nucleic acid from a sample, said method comprising contacting said sample with a detergent and a solid support, whereby soluble nucleic acid in said sample is bound to the support, and separating said support with bound nucleic acid from the sample. Where the method of the invention is used to isolate DNA, it may conveniently be coupled with a further step to isolate RNA from the same sample.

INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/GB 95/02893

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 25912 (MEDICAL RESEARCH COUNCIL) 23 December 1993 *see the whole patent* ---	1-18
X	JOURNAL OF APPLIED BACTERIOLOGY, vol. 74, 1993, pages 78-85, XP002007385 K. SMALLA ET AL.: "Rapid DNA extraction protocol from soil for polymerase chain reaction mediated amplification" *see the whole article* --- -/--	1-18

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
3 July 1996	02. 08. 96

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